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THE SUPPOSED ROLE OF MICROBIOLOGICAL AEROSOL STABILIZERS AS SUBSTITUTES FOR BOUND WATER: AN IN VITRO MODEL SYSTEM

Myron B. Sokolowski
Edward J. Weneck
David Trkula
J. B. Bateman

MARCH 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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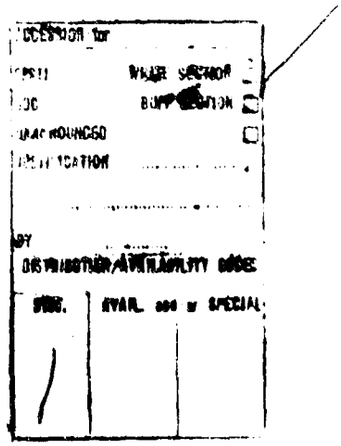

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Physical Science Division
BIOLOGICAL SCIENCES LABORATORIES

Project 1B061102B71A

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ABSTRACT

To test a current belief that inositol can take the place of water in maintaining the stability of desiccated cells, the effect of inositol on the reversible association-dissociation reaction of tobacco mosaic virus protein in 0.06 M phosphate buffer, pH 6.5, was studied by turbidimetric analysis. Addition of inositol lowered the temperature at which the association takes place. Enthalpy (H^o) increased from the control value of +196 kcal $mole^{-1}$ to +260, +273, and +300 kcal $mole^{-1}$ in the presence of 0.10, 0.15, and 0.20 M inositol, respectively, while entropy (S^o) increased from the control value of +600 cal $mole^{-1}deg^{-1}$ to +870, +920, and +960 cal $mole^{-1}deg^{-1}$, respectively. In view of these results, it seems unlikely that inositol displaces bound water, for this would be expected to cause the equilibrium to shift in favor of the dissociated protein at a given temperature and thus to decrease ΔH^o and S^o. A less direct effect seems likely, such as, for example, a conformation change brought about by binding of inositol at positions adjacent to the site of the association reaction.

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TABLE

1. INTRODUCTION*

The loss of viability of bacteria and viruses in aerosol form is retarded by certain sugars, but according to Webb[1] the stabilizing effect of inositol (1,2,3,4,5,6-cyclohexanehexol) is superior to that of the sugars. In attempting to explain this observation, Webb postulates that inositol is bound by the macromolecules of the microorganisms and, because the hydrogen and hydroxyl groups of inositol "are almost identical, physicochemically, to water, . . . the binding of water to inositol would result in a structure similar to that of water-water blocks."[1]

We have thought that Webb's hypothesis might very well be tested critically by examining the effect of inositol on some protein reaction known to involve displacement of "bound" water. Recent work[2-9] indicates that the spontaneous endothermic association of tobacco mosaic virus protein (TMVP) may be such a reaction. Therefore the effect of inositol upon this reaction was studied.

The protein of tobacco mosaic virus (TMV) can be prepared by acetic acid degradation of the virus. At about 10 C in 0.06 M phosphate buffer, pH 6.5, the purified protein exists as an oligomer of three to six associated protein molecules. At room temperature it aggregates to form a hollow rod of high molecular weight, morphologically similar to the native virus. Lauffer and co-workers[2,3,7] showed by turbidimetry that this association reaction is reversible and that it is accompanied by a net entropy increase of 680 cal $mole^{-1}deg^{-1}$. The results of turbidimetric,[2,3,7] dilatometric,[6] and gravimetric[5] experiments support the view that this increase of entropy reflects the release of 130 moles of water per mole of associating protein.

The present paper presents preliminary data on the effects of inositol upon the spontaneous association-dissociation reaction as observed by turbidimetry.

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II. MATERIALS AND METHODS

A. VIRUS

The common strain of TMV was prepared from infected tobacco leaves by the method of Boedtker and Simmons[10] and was purified by differential centrifuging. The purified virus was suspended in demineralized water and stored at 2 to 5 C. Preparations of TMV so prepared displayed typical ultraviolet absorption (peak at 260 nm, trough at 250 nm) and a single boundary in the analytical ultracentrifuge.

B. PROTEIN

The "coat protein" (A-protein) of TMV was prepared free of nucleic acid by the technique of Fraenkel-Conrat[11] and was purified by centrifuging. The protein preparations were dissolved in 0.06 M phosphate buffer at pH 6.5. The ultraviolet absorption maximum was at 280 nm and the minimum at 250 nm. The ratio E_{280}/E_{260} was used to check for contamination with nucleic acid; preparations were considered acceptable if the ratio was >2.4. Preparations that appeared nonhomogeneous in the analytical ultracentrifuge were rejected. The molecular weight was within the reported range, values ranging from those of trimer to hexamer (55,000 to 100,000) obtained using the Yphantis high speed equilibrium method.[12]

In the experiments with inositol, the desired amount of dry inositol was added to the protein stock solution in phosphate buffer. The resultant solution was then dialyzed for about 24 hours against a 50-fold excess of an inositol solution of the same concentration in the same buffer; the dialyzate was centrifuged before use.

C. CONCENTRATION DETERMINATIONS

Concentrations of TMV and TMVP were determined with a Cary 11 spectrophotometer* and were calculated from the optical densities at the respective maxima at 260 and 280 nm, respectively, using the extinction coefficient of 2,700 cm^2g^{-1} for TMV reported by Fraenkel-Conrat and Williams[13] and 1,300 cm^2g^{-1} for TMVP reported by Stevens and Lauffer.[5] The solvent used for determining the protein concentration was 0.033 M phosphate buffer at pH 7.0, as the protein does not associate in this buffer at room temperature.

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* Cary Instruments, 2724 S. Peck Road, Monrovia, Calif. 91016.

D. EXTENT OF ASSOCIATION

The extent of association of TMVP was determined from turbidity measurements at 320 nm in a Cary 11 spectrophotometer; matched 1-cm cuvettes were used. Following the method of Smith,[3] the measurements were made at 320 nm in order to obtain maximum sensitivity while avoiding consumptive absorption.

Turbidity versus temperature curves were obtained by equipping the cuvette compartment of the Cary 11 with thermospacers through which water was circulated from a bath of adjustable temperature. Temperatures in the bath were regulated by a Bronwill thermoregulator* and the temperature of the solution in the cell was sensed by a Teletherm thermistor** calibrated against a NBS thermometer. The precision was ±0.5 C.

The experimental procedure involved diluting the protein solution to about 1×10^{-3} g ml^{-1} and centrifuging at 30,000 rpm at 5 C for 2 hours. The protein was then transferred to an ice bath and subsequently transferred to cold cuvettes. The association was followed by observing the increase of turbidity as the temperature was increased stepwise from 5 C to 21 C. Between 5 C and 13 C there is a base line plateau where no association occurs. After reaching 21 C, the protein solutions were slowly cooled in order to check the reversibility of the reaction. The protein was considered to be at equilibrium at any given temperature when no change in optical density was observed over a period of time. The absence of detectable hysteresis in the association-dissociation curves (Fig. 1) assured us that equilibrium conditions were met.

E. THEORY AND TREATMENT OF DATA

The analysis of the data followed the procedure of Smith and Lauffer.[4,7] Ansevin and Lauffer[5] showed that the kinetics of TMVP association can be approximated by Flory's theory of linear condensation polymerization.[13] Implicit in this theory is the assumption that the reactivity of the functional groups is independent of the size of the polymer. In Flory's treatment[13] the extent of polymerization is given by a parameter p, the fraction of the functional groups initially present in the monomers that have taken part in the association reaction at a given instant. Statistically, the weight average molecular weight, $\bar{M}_w$, of the polymer distribution for any value of p is then $M_o[(1 + p)/(1 - p)]$, where M_o is the monomer molecular weight.

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* Bronwill Scientific Division, Will Scientific, Inc., 277 N. Goodman St., Rochester, N.Y. 14607.

** Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio.

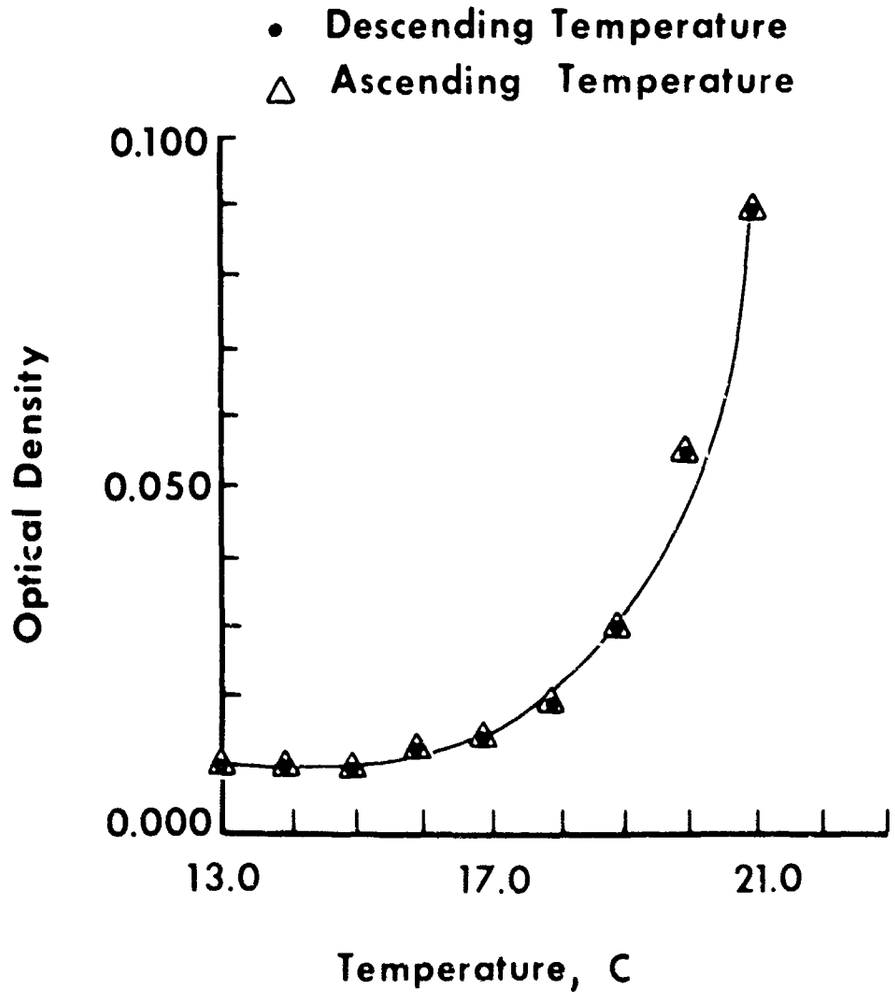

FIGURE 1. Endothermic Association-Dissociation Curve for Tobacco Mosaic Virus Protein. Coincidence of ascending and descending values of the optical density at a given temperature, together with absence of hysteresis, indicates equilibrium conditions.

At sufficiently low protein concentrations, the optical density or extinction E is equal to $2.303\ HcM_n$, where H is an optical factor and c is the concentration in g ml^{-1}. (See, for example, Edsall.[3]) Thus

$$E = HcM_o(1 + p)/(1 - p) = E_o(1 + p)/(1 - p) \qquad (1)$$

Using equation (1) and assuming M_o to be 105,000, one finds that E_o for a solution of concentration 0.88×10^{-3} g/ml would be only about 0.002 (Smith[4]).

This treatment is valid only in dilute solutions and in cases where the particles scattering the light are considerably smaller than the wavelength of the incident light. Another restraint on the theory is that, according to Flory,[2,4] the condensation kinetics apply only to conditions where $p<0.9$. According to Smith,[4] the maximum length of the aggregated protein under these conditions is about 400 Å or <0.15 λ. It is thus reasonable to assume that the treatment is at least empirically sufficient.

It can be shown[2] that the equilibrium constant, K, for the monomer $\rightleftharpoons$ polymer reaction at a given temperature can be expressed as the ratio of a first order dissociation reaction, k_1, and a second order association constant, k_2, and that it can be related to the parameter p by the expression[3]

$$K = k_1/k_2 = p/(1 - p)^2c \qquad (2)$$

From elementary thermodynamics, one can then calculate the standard free energy, enthalpy, and entropy of the reaction:

$$\Delta F^o = -RT\ln K \qquad (3)$$

$$F^o = H^o - T S^o \qquad (4)$$

Hence a plot of $\ln[p/(1 - p)^2]$ versus $1/T$ yields the H^o and S^o terms:

$$\ln[p/(1 - p)^2] = \ln c + (S^o/R) - (H^o/RT) \qquad (5)$$

The slopes H^o/R were calculated by least squares from these graphs.

Keeping in mind that the calculated optical density of the "monomeric" protein solution is about 0.002, and referring to Figure 1, one can see that the plateau at lower temperatures (where no association occurs) is considerably higher than the predicted optical density within this temperature range. This phenomenon was also observed by Smith: "The initial low temperature optical densities measured were almost always too high as judged from theoretical calculations of the turbidity expected from particles of the 'monomer' size presumed to be present. For this reason, the measured initial optical densities were corrected by the amount required to give the theoretical optical density for particles of A-protein size, that is, for particles of molecular weight about 100,000."[4] This is considered a serious drawback because the lower values of p are very sensitive and critical in the analysis used. However, the suggested procedure was followed in the present work.

III. RESULTS

A. TMVP IN 0.06 M PHOSPHATE BUFFER, pH 6.5

Figure 2 (solid square symbols) shows the change of the corrected optical density with increasing temperature. The result of van't Hoff treatment of these data is shown in Figure 3. The enthalpy change calculated from the least squares slope of the data in Figure 3 is 196,000 cal $mole^{-1}$, in agreement with Smith.[4] Values for ΔF^{o}, -4,200 cal $mole^{-1}$, and for ΔS^{o}, +600 cal $mole^{-1}deg^{-1}$, were obtained for the reaction at 19 C, the temperature at which Smith calculated his values. Smith's values at ionic strength 0.1 were as follows: F^{o} = -9,360 cal $mole^{-1}$; H^{o} = 190,000 cal $mole^{-1}$; S^{o} = 682 cal $mole^{-1}deg^{-1}$.

B. TMVP IN 0.06 M PHOSPHATE BUFFER, pH 6.5, AND INOSITOL

Figure 2 shows the effects of inositol at three concentrations on the increase of optical density with rising temperature. Increasing concentrations of inositol from 0.1 to 0.2 M appear to favor association at lower temperatures. Figures 4, 5, and 6 show the van't Hoff plots of the data; the derived thermodynamic values are summarized in Table 1.

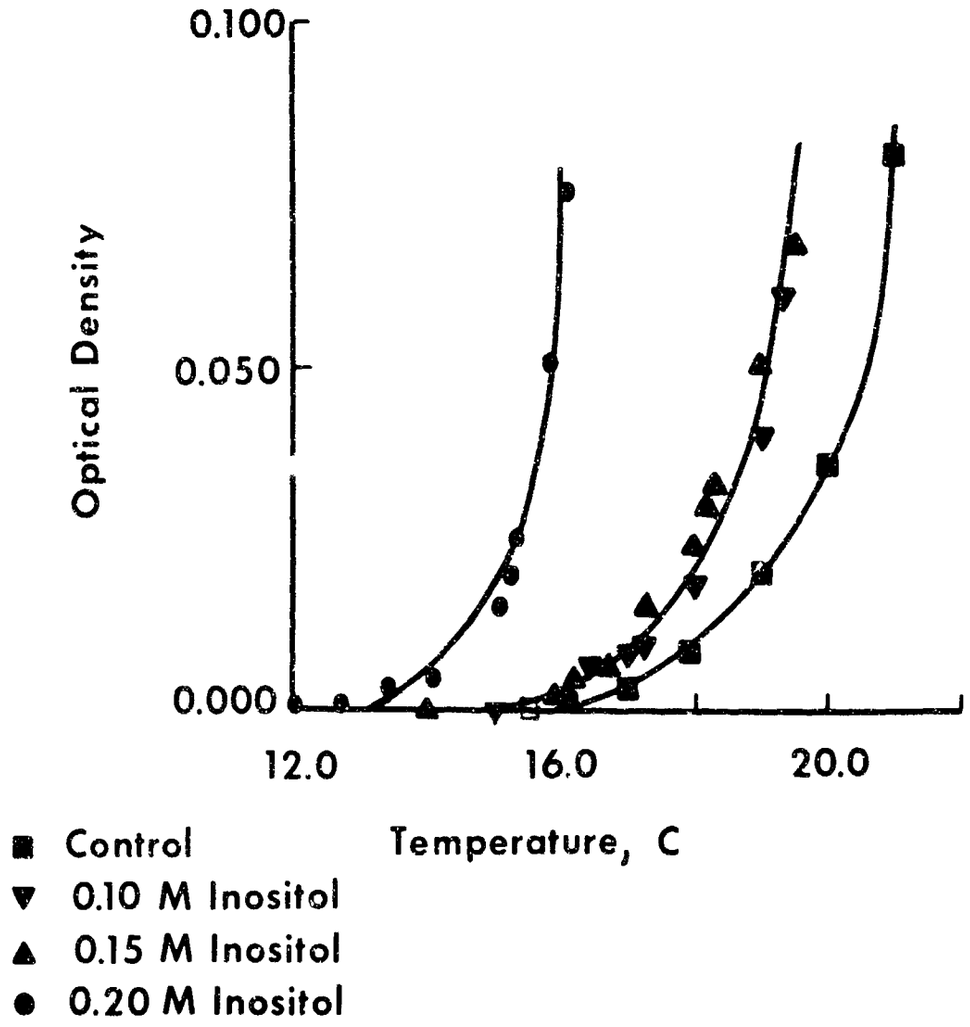

FIGURE 2. Effect of Inositol on the Association-Dissociation of Tobacco Mosaic Virus Protein.

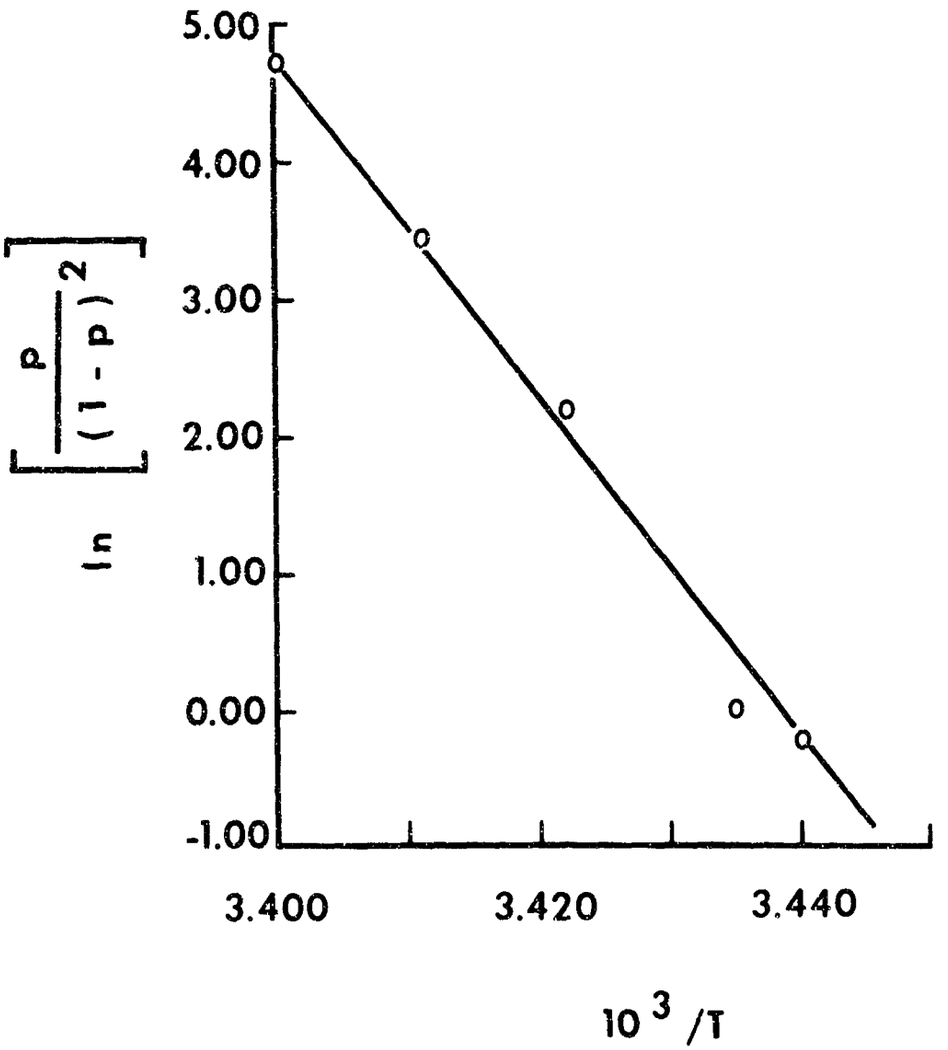

FIGURE 3. Van't Hoff Treatment of Data for TMV Protein in 0.06 M Phosphate Buffer, pH 6.5. $\Delta H^o = +196{,}000$ cal mole^{-1}.

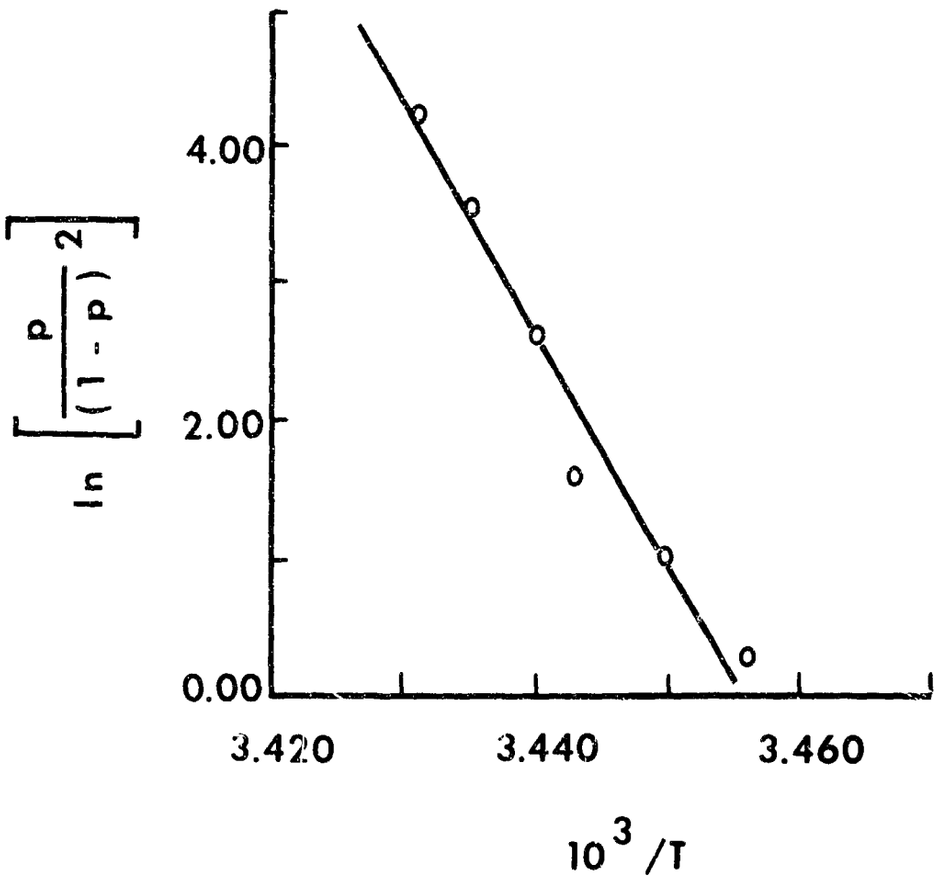

FIGURE 4. Van't Hoff Treatment of Data for TMV Protein in Stock Buffer Solution and 0.10 M Inositol. ΔH° = +260,400 cal $mole^{-1}$.

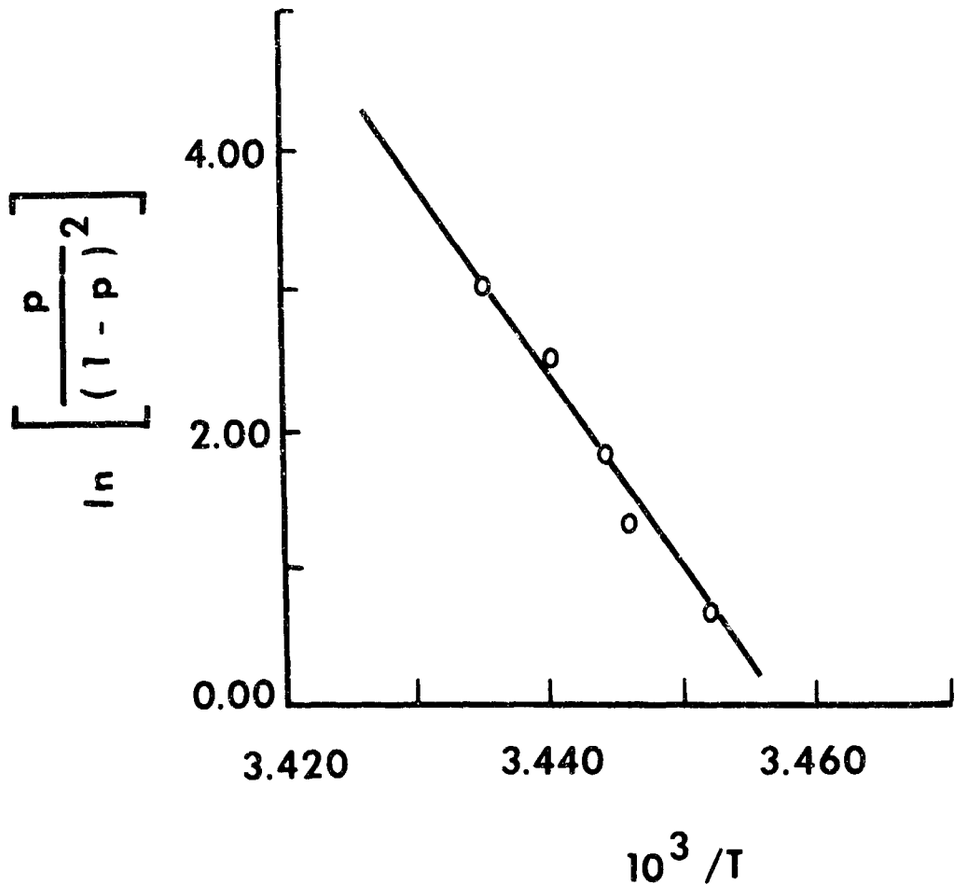

FIGURE 5. Van't Hoff Treatment of Data for TMV Protein in Stock Solution and 0.15 M Inositol. ΔH^{o} = +272,900 cal mole^{-1}.

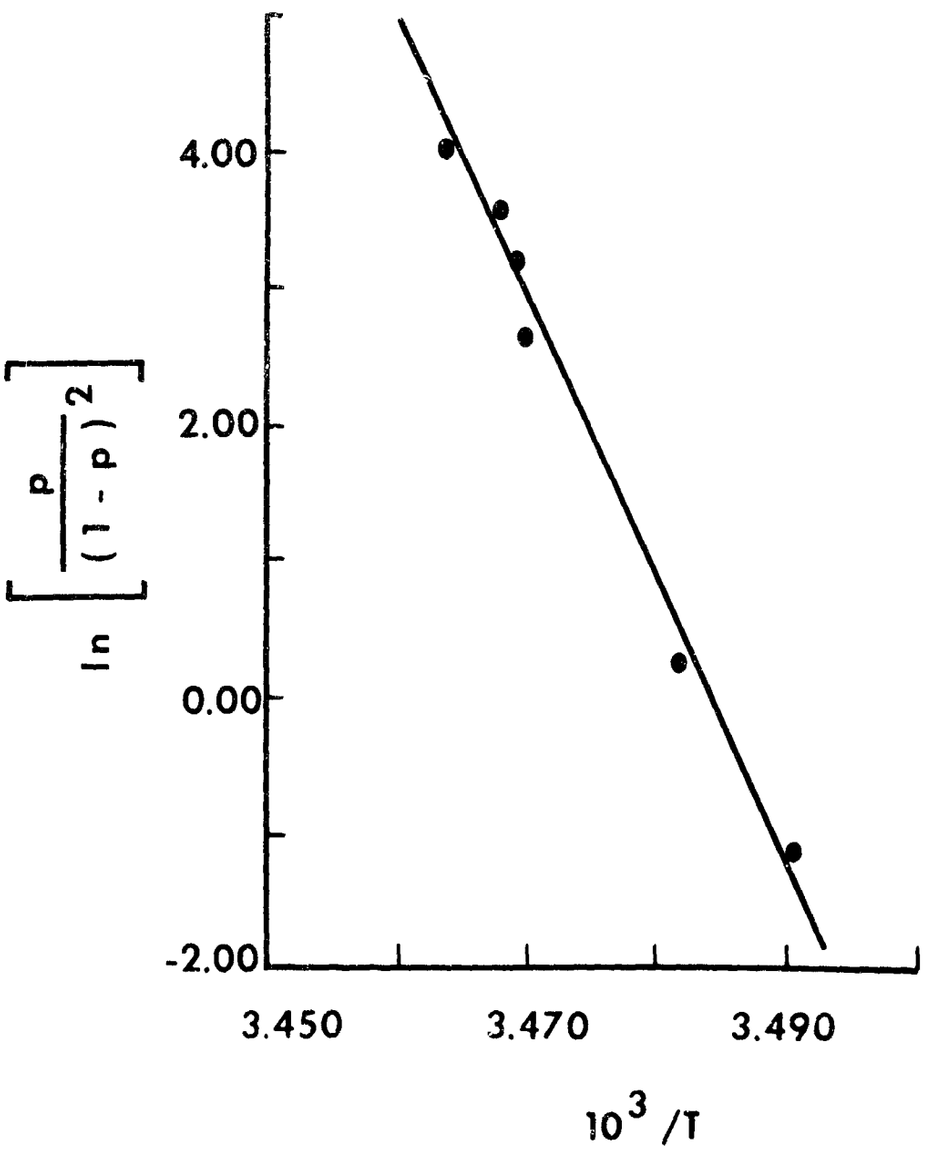

FIGURE 6. Van't Hoff Treatment of Data for TMV Protein in Stock Solution and 0.20 M Inositol. H^o = +300,000 cal $mole^{-1}$.

TABLE 1. EFFECTS OF INOSITOL AND SUCROSE ON THE THERMODYNAMIC PARAMETERS OF TMVP EQUILIBRIUM AT 19 C[a]

Additive	Concentration of Additive, M	ΔH^o, cal mole^{-1}	F^o, cal mole^{-1}	S^o, cal mole^{-1}deg^{-1}
Inositol	0.00	196,000	-4,200	+600
	0.10	260,000	-6,000	+870
	0.15	273,000	-6,000	+920
	0.20	300,000	-10,200	+960
Sucrose[b]	0.00	225,000		+860
	0.20	262,000		+936

a. Reaction in 0.06 M phosphate buffer, pH 6.1.
b. Data for sucrose from Shalaby and Lauffer.[8]

IV. DISCUSSION

TMVP undergoes spontaneous, reversible, endothermic association. Lauffer and colleagues have suggested[3,5-8,9] that the TMVP subunits are more highly hydrated than the polymer, and that the thermodynamic constants of the reaction can be accounted for by assuming that the association can occur only by the displacement of some of the bound water, a process assumed to be thermodynamically equivalent to the melting of a comparable amount of ice.

The present experiments have yielded thermodynamic data very similar to those of Smith[4] and have shown in addition that in the presence of inositol the association is favored. The increases of enthalpy and of entropy are augmented by about one-third, and the decrease of free energy is more than doubled. Qualitatively similar, but less marked, effects have been noted in the presence of sucrose by Shalaby and Lauffer[8] (Table 1). These authors suppose that the effect of sucrose, which is thought to promote the transition of water to one of its more highly ordered forms, can indirectly modify the behavior of a protein by perturbing its interactions with the solvent. It is not obvious to us that such indirect effects could be brought about without gross modification of the water activity, and we prefer, for the time being, to attribute the effect of sucrose to binding

of this substance in the vicinity of the association centers. Conformational change in the protein molecule consequent upon binding of sucrose has been demonstrated recently in the case of β-lactoglobulin by Clement-Metral and Yon.[16] In our experiments the pronounced effect of inositol could, of course, be produced by a similar mechanism, the details of which must remain unresolved. In any event, the observed effects of inositol are qualitatively the opposite of those that might be expected from Webb's hypothesis of the enhanced binding of a simulated water substance as the basis for the stabilizing effect of inositol on dehydrated microorganisms.

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13. ABSTRACT

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14. Key Words
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*Stabilizat...
Sugar alcohc
Tobacco mosa. virus
Phosphates
Proteins
Models

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